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Efficacy and Kinetics of Glycerol Inactivation of HIV-1 in Split Skin Grafts

Paul U. Cameron,[4] Joanne C. Pagnon,[1] Jeroen van Baare,[3] Jeanette C. Reece,[4] Nicholas J. Vardaxis,[2] and Suzanne M. Crowe[1]*

[1]*AIDS Pathogenesis Research Unit, Macfarlane Burnet Centre for Medical Research, Victoria Australia*
[2]*Department of Medical Laboratory Science, Royal Melbourne Institute of Technology, Victoria, Australia*
[3]*Euro Skin Bank, Dutch Burns Foundation & Burns Centre, Red Cross Hospital, Beverwijk, The Netherlands*
[4]*Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia*

Allogeneic split skin grafts are used widely in the treatment of burns. The relative simplicity of glycerol preservation of skin suggests it will be used increasingly in areas of high HIV-1 seroprevalence. The ability of glycerol preservation to inactivate HIV-1 present in skin graft infected *in vitro* was determined using a macrophage tropic strain HIV-1 as a cell-free virus suspension, within infected PBMCs, or within *in vitro* HIV-1 infected fresh cadaveric split skin. Different temperatures and concentrations of glycerol were used and infectivity determined by coculture with mitogen activated peripheral blood mononuclear cells (PBMCs) and measurement of reverse transcriptase activity after 7–10 days. Cell-free HIV-1 was inactivated within 30 min at 4°C in glycerol concentrations of 70% or higher. During similar exposure cell- or skin-associated HIV-1 titer was reduced but not eliminated with 70% and 85% glycerol at 4°C. HIV-1 was recovered consistently from skin stored in 85% glycerol at 4°C for up to 72 hr but virus isolation was infrequent after storage for more than 5 days. At 20°C or 37°C, 70% or 85% glycerol could inactivate cell- or skin-associated HIV-1 within 8 hr. The initial glycerolization procedures and the storage at 4°C eliminated effectively HIV-1 from skin. ***J. Med. Virol. 60:182–188, 2000.***

KEY WORDS: virus inactivation; glycerol; skin graft

INTRODUCTION

Cryopreservation and glycerol treatment have been used for long-term storage of cadaver skin for use as allografts. Although donor screening for an increasing range of pathogens has greatly reduced the risk of transmission of human immunodeficiency virus (HIV) and other microbes [Eastlund, 1995], human to human transmission of infectious agents via transplantation of bone, semen, cornea, heart valves, marrow, and skin has been reported [Clarke, 1987; Eastlund, 1995; McMaster and Mirza, 1995]. Human immunodeficiency virus type 1 (HIV-1) transmission by transplantation of organs and tissues has occurred usually where donors were not tested for HIV antibodies or the organs and tissues were used before the results of serology were known. HIV transmission has also occurred following transplantation of fresh organs but not lyophilized tissue from a seronegative but HIV-infected multi organ donor [Simonds et al., 1992]. One case of HIV transmission from allograft skin has been reported when fresh skin was used before serological results were available [Clarke, 1987].

High concentrations of glycerol (85%) have been used for many years to preserve tissues used for transplantation. It has been shown that glycerol preservation maintains cellular and structural morphology [Wolff and Dienemann, 1990; Richters et al., 1996], may reduce the immunogenicity of the tissue [Hettich et al., 1994; Richters et al., 1996] and has antimicrobial properties [van Baare et al., 1994; Marshall et al., 1995]. Intracellular herpes simplex and poliovirus were found to be inactivated when infected cells were exposed to 98% glycerol [Marshall et al., 1995]. However, the effect on viruses of prolonged storage at 4°C and the entire glycerolization procedure used for cadaver skin, in which the glycerol concentration is slowly increased from 50% glycerol to 85% glycerol with two incubation steps of 3 hr at 33°C, has not been determined [de Backere, 1994]. Skin can contain HIV-infected cells, including Langerhans cells [Zambruno et al.,

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*Correspondence to: Suzanne M. Crowe, AIDS Pathogenesis Research Unit, Macfarlane Burnet Centre for Medical Research, Yarra Bend Rd., Fairfield, Victoria, Australia 3078.
E-mail: tolli@burnet.edu.au

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1991; Compton et al., 1996] and keratinocytes [Ramarli et al., 1995], both of which are able to transmit infection to T cells. Langerhans cells and dermal dendritic cells carrying HIV-1 are considered to be of major importance in the transmission of infectious virus to T cells [Reece et al., 1998] and restrict transmitted virus to those strains that can use the CCR5 coreceptor [Zaitseva et al., 1997].

The relative simplicity and modest infrastructural requirements of the current glycerolization protocols compared to cryopreservation make it attractive for developing countries where there are high frequencies of HIV-1 infection and limited resources for extensive testing of seronegative donors for recent HIV-1 infection. This study was undertaken to investigate whether HIV as free virus, in single cells or cadaveric skin infected exogenously could be effectively inactivated during the current processes of glycerolization used in skin banking and to assess the risk of transmission that might be associated with transplantation of treated skin.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were obtained from fresh buffy coat cells by centrifugation over ficoll hypaque (Pharmacia-Biotech, Uppsala, Sweden) columns. PBMCs were activated for 3 days with staphylococcal enterotoxin B (10 ng/ml SEB, Sigma, St. Louis, MO) or phytohaemagglutinin (PHA 10 ug/ml, Murex Diagnostics Ltd, Maidenhead, England) in RPMI 1640 (Cell Image, Sydney, Australia) supplemented with 10% heat inactivated foetal calf serum (PA Biologicals, Sydney, Australia), 2mM glutamine (Gibco BRL, Grand Island, NY) and 24 ug/ml gentamicin (DBL, Bentley, Australia) (RF10). Stimulated PBMCs at 1×10^5/well were incubated with ten-fold dilutions of 85% glycerol (BDH, Kilsyth, Australia) to determine cellular toxicity. After seven days incubation in RF10 with 5% recombinant interleukin 2 (IL-2, Boehringer-Mannheim, Mannheim, Germany), cells were pulsed for 12 hr with tritiated thymidine (Amersham Life Science, Buckinghamshire, UK), harvested with a cell harvester (Tomtec harvester 96), and incorporation determined with a micro beta counter (LKB, Wallac, Turku, Finland).

Virus Culture

HIV-$1_{Ba\text{-}L}$ is macrophage tropic and infects epidermal and subepidermal cells [Palacio et al., 1994] including dendritic cells [Cameron et al., 1996] with greater efficiency than T cell line tropic virus. Stocks of HIV-1 were produced in PHA-stimulated PBMCs maintained in culture medium containing 10% fetal calf serum and 5% IL-2 for 14 days at 37°C and filtered (0.2 μm) before storing at −70°C.

Reverse Transcriptase (RTase) Assay

Culture supernatants (10 μl) were collected into microtitre plates with an equal volume of 0.3% NP40 and stored frozen until batch processed. The method described previously [Willey et al., 1988] was used with minor modification. The reactions were carried out with 0.5 μCi ^{33}P-dTTP (Amersham, UK) for 3–4 hr at 37°C and 6 μl of the reaction spotted onto DE81 chromatography paper (Whatman, Maidstone, UK) and air dried. Dry filters were washed 6 times with 2×SSC buffer (0.3 M sodium chloride and 34 mM sodium citrate). The dried filters were then spotted with Meltilex scintillant (Wallac, Finland), covered with plate sealers and counted on a micro beta counter. Values greater than the mean plus three times the standard deviation of the value for culture media from uninfected controls were considered positive.

Tissue Culture Infectious Dose Assay ($TCID_{50}$)

Tissue culture infectious dose assay ($TCID_{50}$) was determined by adding 1×10^5 SEB-stimulated PBMCs/well to replicate 10-fold dilutions of virus in a total of 200 μl/well of culture media. Half media changes were undertaken twice weekly. Reverse transcriptase assays were performed on culture supernatant and $TCID_{50}$ was calculated by the Reed-Muench method [Reed and Muench, 1938].

Glycerol Treatment of Virus, Cells and Skin

Glycerol (BDH, Australia) was diluted in phosphate buffered saline (PBS) to give concentrations of 50%, 70% and 85%. Glycerol is toxic for cells including those needed to test virus infectivity in biological assays. Direct glycerol toxicity was avoided by treating concentrated virus solutions and then diluting the glycerol containing virus solutions to levels at which the glycerol was nontoxic for the coculture cells. All cellular detection assays were carried out at glycerol concentrations of less than 0.85%, a concentration found not to inhibit proliferation of activated T cells (data not shown).

Physical agents such as glycerol that may be effective as antiviral agents may interfere with the biological detection system [Gordon et al., 1993]. The high density of glycerol ($d = 1.25$ g/ml) may reduce cell recovery and the ability of HIV-1 to bind to cellular targets. Thus washing procedures were optimized and cell losses occurring during centrifugation through glycerol solutions monitored by determining the recovery of radiolabelled cells treated in parallel. Radiolabelled cells were prepared by incubation of PBMCs activated for of 3 days with SEB (50 ng/ml, Sigma) for 18 hr in ^{3}H-thymidine at 20 μCi /ml.

Inactivation of Extracellular HIV-1

Virus was pelleted from stock culture supernatant (1.4×10^6 $TCID_{50}$/ml of HIV-$1_{Ba\text{-}L}$] at 17,000 rpm for 1 hr at 4°C in a Biofuge 22R (Heraeus Sepatech). A volume of 300 μl of 50%, 70%, and 85% glycerol or PBS was added to the pellet and incubated according to skin bank protocols; 50% glycerol or PBS for 2 hr at room temperature (RT), 70%, 85% glycerol or PBS for 3 hr at 37°C. After incubation the glycerol was diluted by add-

ing 700 μl of PBS. The virus was centrifuged at 17,000 rpm for 1 hr at 4°C. After aspiration of 950 μl of supernatant the remaining 50 μl was adjusted to 600 μl with RF10/5% IL-2. The end-point titrations were performed using RTase assay of day 10 supernatants; the $TCID_{50}$ were calculated by the Reed-Muench method. For the kinetic analysis concentrated virus was added to glycerol solutions and 10 μl of the mixture incubated in each well of a microtitre plate at the temperature and glycerol concentrations tested. At the end of the incubation 300 μl of medium was added to each well and 100 μl of solution added to 200 μl of medium containing 1×10^5 activated T cells. Virus production was measured by RTase production.

Inactivation of Cell-Associated Virus

SEB stimulated PBMCs were infected with HIV-$1_{Ba\text{-}L}$($1.42\times10^6 TCID_{50}$/ml) for 7 days and exposed to varying concentrations of glycerol in 96 well plates. The glycerol was removed by washing 5× in PBS. The infected cells were resuspended in 200 μl RF10/5% IL-2 and 5×10^4 cells were added to an equal number of untreated uninfected stimulated PBMCs. An RTase assay was performed on supernatants obtained on days 2, 5, 7, and 10. To control for cell recovery during glycerol exposure and washing, uninfected ^3H thymidine-labelled activated PBMCs were treated with glycerol in parallel. Limiting dilution of infected cells in uninfected PBMCs was used to determine changes in viral infectivity.

Virus Inactivation in Split Thickness Skin

Glycerolization procedures for skin and cells were designed to follow the protocol used in skin banking and to investigate the antiviral effect of each component of a commonly used protocol [de Backere, 1994]. In this protocol the freshly harvested skin is placed in 50% glycerol at room temperature during transport. At the skin bank the skin is incubated in 50% glycerol for 2 hr at room temperature. Skin is then incubated sequentially at 33°C in 70% glycerol for 3 hr and 85% glycerol for 3 hr before storing at 4°C in 85% glycerol for at least 2 weeks.

Split thickness skin (0.3 – 0.5mm) was obtained from the Victorian Institute of Forensic Medicine within 24 hr of death. Donors were negative serologically for HIV-1, HTLV-1, hepatitis B and hepatitis C. Skin (1.5-2 × 1.5-2 cm) was infected with 200 μl of HIV-$1_{Ba\text{-}L}$ (3×106 $TCID_{50}$/ml) for 12 hr. Virus was treated with 10 mM $MgCl_2$ and 10 U/ml DNase (Worthington, Freehold, NJ) for 2 hr at room temperature before use. The infected skin was washed with PBS three times and stored in 85% glycerol at 4°C for up to 28 days or treated according to the glycerolisation protocol [de Backere, 1994] but incubated at a more convenient temperature of 37°C rather than 33°C. After storage, glycerol was removed by washing with PBS (5 washes for 5 min each) and the skin was cocultured with uninfected 3 day SEB-stimulated PBMCs.

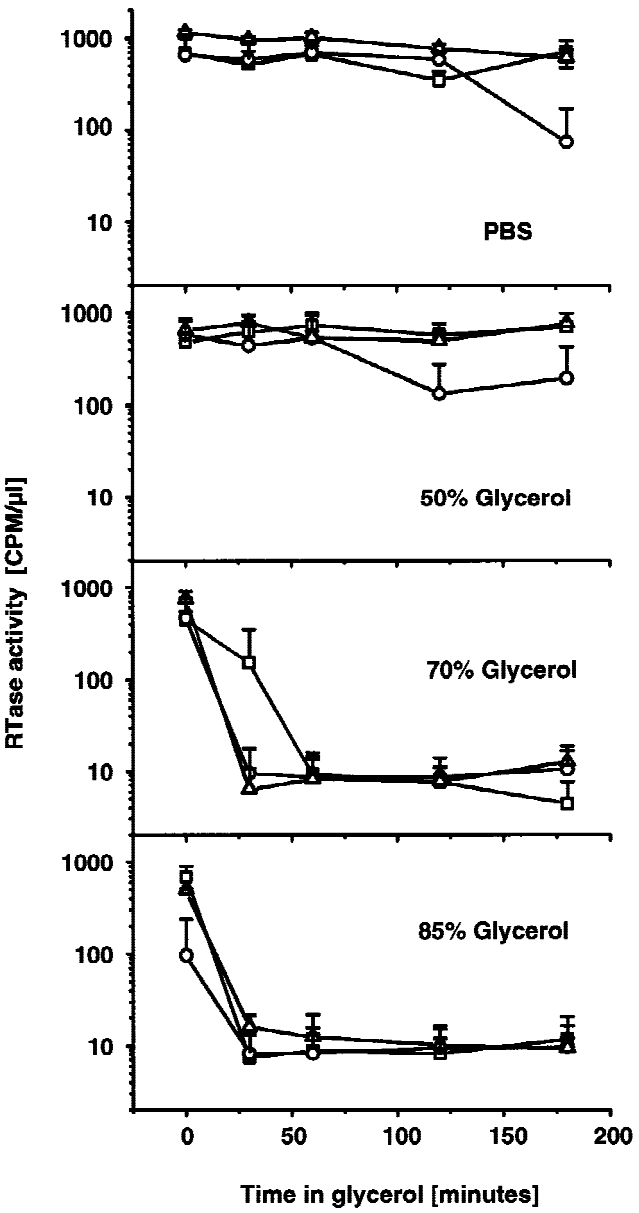

Fig. 1. Kinetics of inactivation of cell-free HIV-1 by glycerol. HIV-$1_{Ba\text{-}L}$ was concentrated by centrifugation and exposed to varying concentrations of glycerol or phosphate buffered saline (PBS) at 4°C **(open square)**, room temperature **(open circle)** or 37°C **(open triangle)** for the indicated times. The mean and standard deviation (SD) of the RTase activity in the culture supernatant at each time point are shown.

RESULTS

Inactivation of HIV-1 Virions by Glycerol

The kinetics of HIV-1 inactivation were determined using virus concentrated from culture supernatants from HIV-1 infected cells (Fig. 1) . Glycerol at a concentration of 50% had little effect on the recovery of HIV-1 at 4°C, room temperature or 37°C . Glycerol at 70% or 85% inactivated HIV-1 within 30–60 min at all three temperatures. At high glycerol concentration there was no effect of temperature in that treatment

TABLE I. Quantitation of Glycerol Inactivation of Cell-Free HIV-1 and Cell-Associated HIV-1*

	PBS	50% glycerol	70% glycerol	85% glycerol
0 hours	$10^{3.32}$	$10^{2.5}$	$10^{1.5}$	$10^{2.5}$
	[a][0]	[0.82]	[1.82]	[0.82]
2 hours	10^{4}	10^{2}	nd	nd
	[0]	[2]		
3 hours	$10^{4.46}$	nd	$10^{0.5}$	10^{0}
	[0]		[3.96]	[>4.46]
	PBS	50% glycerol	70% glycerol	85% glycerol
0 hours	12,883	6,918	25,703	3,801
	[b][0]	[0.27]	[0.30]	[0.53]
2 hours	12,883	371	nd	nd
	[0]	[1.54]		
3 hours	12,883	nd	78	<66
	[0]		[2.13]	[2.29]

*HIV-1 titre [$TCID_{50}$] and log reduction after the indicated treatment and incubation time. See methods for titration protocol. Number of infected cells/10^6 PBMCs and log reduction after glycerol treatment. The frequency of cells able to transmit infection was determined by limiting dilution. See methods for details of protocol.
[a]Log reduction in virus titre at each incubation point relative to PBS control for the same time point.
[b]The log reduction in frequency of infected cells as the proportion of the PBS control at that time point. nd = not done.

with 85% glycerol at any temperature (4–37°C) completely eliminated infectious virus.

The reduction in HIV-1 titre during incubation in glycerol was measured by a limiting dilution assay at each of the glycerol concentrations used in the sequential steps in the standard protocol [de Backere, 1994] (Table IA). No virus was recovered following the 85% glycerol treatment (reduction in virus $TCID_{50}$ of $>10^{4.46}$). The viral $TCID_{50}$ was reduced after 50% glycerol for 2 hr at room temperature (reduction in viral titer of 10^2) and 70% glycerol at 37°C for 3 hr (reduction in viral titer of $10^{3.96}$). The minimal cumulative reduction in titre of free virus expected for the initial phase of the glycerolization protocol ($10^{10.76}$) was estimated from the sum of the log reduction occurring in each of these component steps.

Inactivation of Cell-Associated HIV-1

Glycerol treatment of HIV-1 infected cells using the ESB protocol [de Backere, 1994] prevented virus transmission to activated PBMCs (Fig. 2A). The treatment with 50% glycerol for 2 hr at room temperature reduced the frequency of infected cells but allowed transmission. Treatment with 70% or 85% glycerol at 37°C for up to 4 hr reduced markedly or eliminated infectious cells. There was no significant difference between the cell recovery in glycerol-treated and untreated cell populations (data not shown). The reduction in recoverable virus at each of these steps was estimated by limiting dilution assay (Table IB). Based on these limiting dilution assays, glycerolization of HIV-1 infected PBMCs was estimated to produce a minimal cumulative reduction of $10^{5.9}$ in the frequency of infected cells.

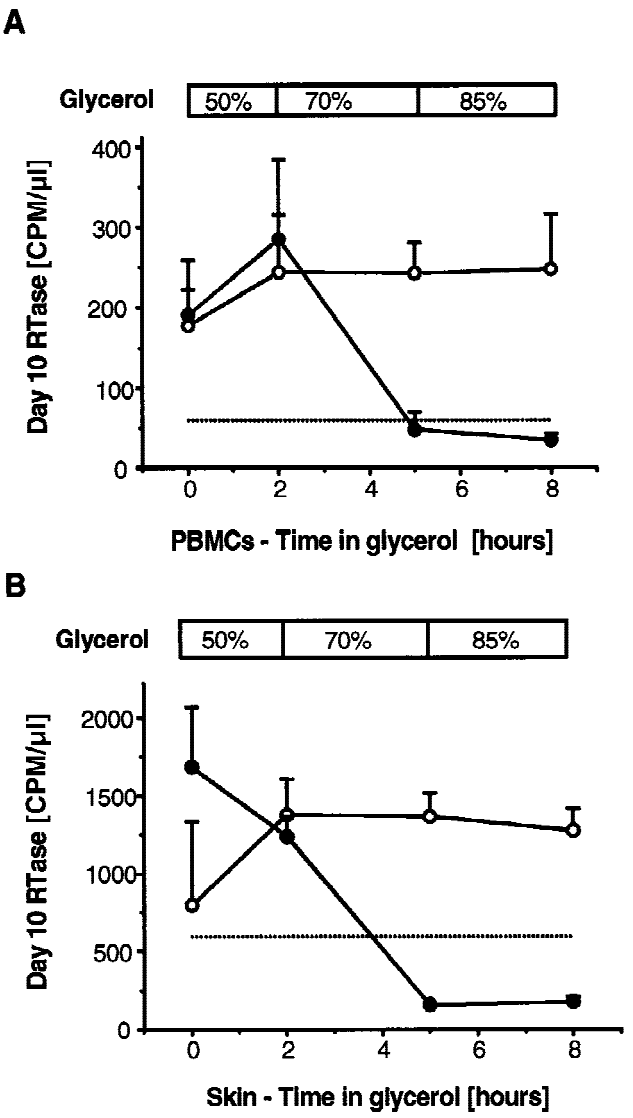

Fig. 2. Quantitation of the infectivity of HIV-1 in glycerol treated HIV-1-infected PBMCs or skin grafts. **(A)** Reduction of infectivity of HIV-1-infected cells by glycerolization. Activated PBMCs were infected with $HIV\text{-}1_{Ba\text{-}L}$ and after 7 days treated with PBS (open circle) or sequentially with 50% glycerol at room temperature for 2 hours, 70% glycerol at 37°C for 3 hours and 85% glycerol at 37°C for 3 hr (closed circle). Mean and SD for RTase in culture supernatants are shown. The cutoff (dotted line) is the mean RT activity present in supernatants of uninfected cells + 3 SDs. **(B)** HIV-1 infected skin was glycerolized with sequential incubation in 50% glycerol at room temperature for 2 h, 70% glycerol at 37°C for 3 hr and 85% glycerol at 37°C for 3 hours. Virus recovery (mean + SD of RTase activity in 10 day culture supernatants) after each step was analysed by coculture of PBS (open circle) or glycerol (closed circle) treated skin with activated PBMCs.

Inactivation of HIV-1 in Exogenously Infected Split Skin

The effect of the initial glycerolization process at room temperature and 37°C was assessed by treating infected skin according to the glycerolization protocol (Fig. 2B). Infectious HIV-1 was effectively eliminated beyond the second step in the protocol (37°C for 2 hr in 70% glycerol). The initial step of 50% glycerol at room

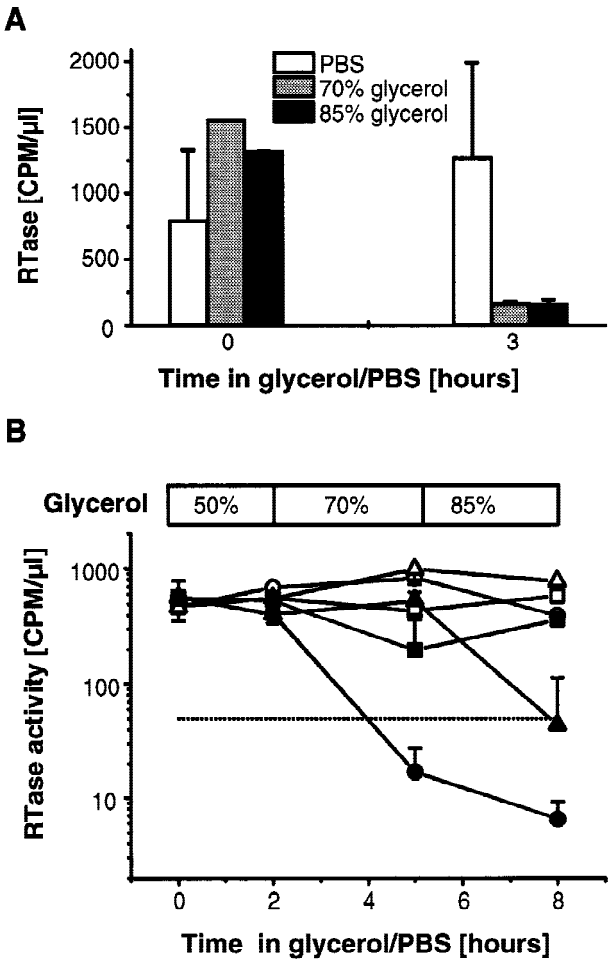

Fig. 3. The effect of temperature of incubation on the inactivation by glycerol of HIV-1 in infected skin. **(A)** Virus inactivation during glycerolization in 70% or 85% glycerol at 37°C. Virus recovery was determined as in Figure 2B. **(B)** Inactivation of skin-associated virus at incubation temperatures greater than 4°C. Exogenously infected skin was treated with PBS (open symbols) or sequentially in glycerol (closed symbols) concentrations of 50%, 70% and 85% at 4°C (square), room temperature (up triangle) or 37°C (circle).

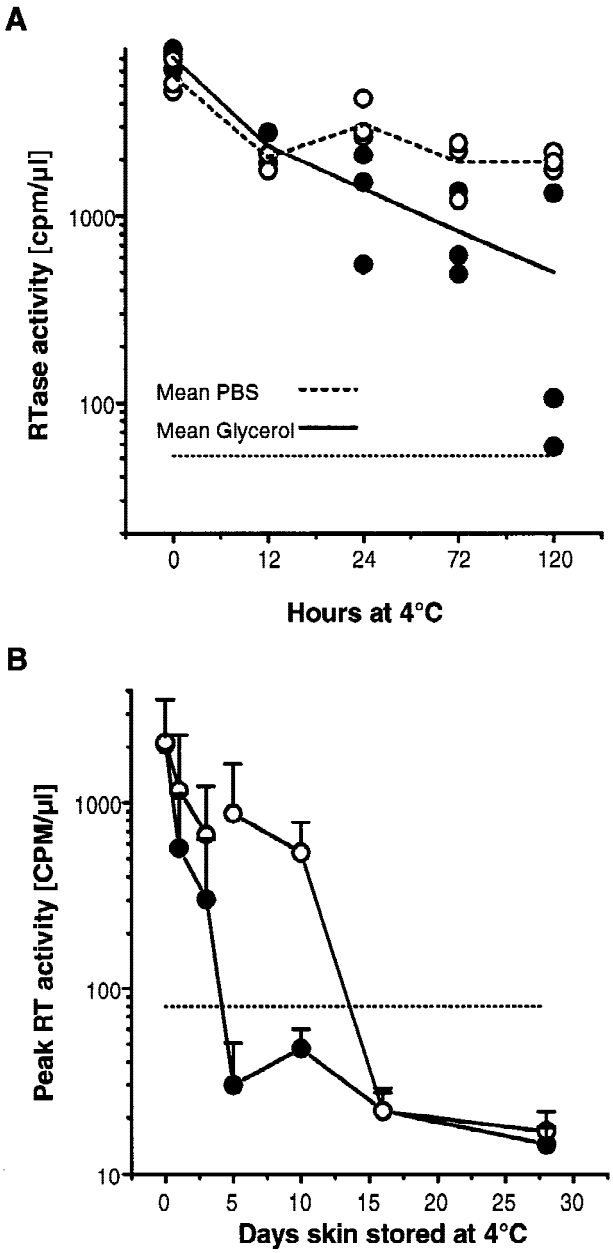

Fig. 4. Inactivation of HIV-1 in infected skin stored at 4°C. **(A)** HIV-1_{Ba-l} infected skin was placed in PBS (open circle) or in 85% glycerol (closed circle) at 4°C. After the indicated intervals of storage the skin was washed, cocultured with activated PBMCs (see methods). The mean and SD of RTase values for triplicate cultures are shown. Storage in glycerol for up to 5 days results in a progressive reduction in RTase activity in the cocultures but some residual recovery occurs up to 5 days. The cutoff (dotted line) is the mean + 3SD of the RTase activity in culture supernatants obtained on day 10 from uninfected cells. **(B)** Recovery of infectious virus in skin is reduced during prolonged storage at 4°C in PBS (open circle) and further reduced by concurrent 85% glycerol exposure (closed circle).

temperature however did not result in significant loss of infectious virus. Incubation in either 70% or 85% glycerol was sufficient to eliminate virus (Fig. 3A). In contrast to the inactivation of free virus, higher temperatures had a marked effect on the virus recovery from split skin (Fig. 3B). Virus was recovered after each step at 4°C, was reduced after the final step at room temperature and was eliminated after the second step (2 hr in 70% glycerol) when performed at 37°C.

Kinetics of Inactivation of HIV-1 in Skin Stored at 4°C

The effect of glycerol inactivation of HIV-1 within infected skin during storage in glycerol was determined by coculture of exogenously infected skin exposed to glycerol for up to 28 days. Virus could be consistently recovered from skin stored in 85% glycerol at 4°C for up to 3 days but only rarely after 5 days of glycerol exposure (Fig. 4A, 3B). No virus was recovered from skin after 10 days of storage in 85% glycerol (Fig. 3B). After 10 days there was also reduced recovery of HIV-1 from skin stored at 4 °C in PBS (Figure 4B).

DISCUSSION

We have shown that glycerol treatment is able to inactivate HIV-1, as free virus as well as cell and skin-

associated HIV-1. The glycerolization procedure used for cells and skin followed the standard protocol [de Backere, 1994] but for laboratory convenience used 37°C rather than 33°C incubation. It is clear from this data that HIV-1 inactivation is dependent on both the concentration of glycerol and the incubation temperature. Although free virus is inactivated rapidly at all temperatures by glycerol at concentrations greater than 70%, infectious virus associated with skin can survive when stored in 85% glycerol at 4°C for up to 5 days.

The physical property of glycerol's high density (d = 1.25 g/ml) may interfere with the biological detection system [Gordon et al., 1993], reducing cell recovery and the ability of HIV-1 to bind to cellular targets. These undesirable effects were minimized by using low concentrations of glycerol in the coculture assays and by controlling for cell loss by using labelled cells. There was little effect of temperature on glycerol inactivation of free virus, with marked reduction observed at 4°C, room temperature, and 37°C using higher concentrations of glycerol. Reduction of viral titre was demonstrated with concentrations of glycerol more than 70% but there was minimal reduction with 50% glycerol. No infectious virus was recovered after exposure to 85% glycerol. Individually the 3 step glycerolization protocol [de Backere, 1994] resulted in reductions of viral titre of 10^2, $10^{3.96}$ and $10^{4.46}$ to give an expected cumulative reduction in the order of 10^{10} for the entire protocol.

Infectivity of HIV-1 infected cells was reduced by glycerol treatment in a similar concentration-dependent fashion. However infected cells could transmit infection to activated T cells at concentrations of glycerol that have been found to eliminate completely viable cells and free virus. The antiviral effects of glycerol on infected split thickness skin infected exogenously was most effective with high glycerol concentrations (70% or higher) at room temperature or 37 °C. HIV-1 could be recovered from infected split skin that were not treated by the glycerolization protocol but stored at 4°C in 85% glycerol for up to 5 days.

The precise mechanism of transfer of virus to PBMCs from cells and tissues including skin that has been treated with glycerol is not clear. Glycerol treated cells are nonviable [Richters et al., 1996], but nonviable infected cells or cellular debris may be able to transmit HIV-1 infection [Kornbluth, 1994]. This transmission requires the presence of phagocytic monocyte/macrophages but these cells would be present in the bed of a skin graft.

It is likely that skin containing high titre virus will be infectious unless treated with concentrations of glycerol of 70% or higher at sufficiently high temperature. Although the ability of glycerol to displace water has been related to its antiviral properties [de Jong et al., 1975] the assays for HIV-1 infectivity that have been used in this and previous studies suggest that simple dehydration of virus supernatants does not eliminate infectious HIV-1 [Hanson et al., 1989] however, after exposure to high concentrations of glycerol for appropriate times, cells and virus are rendered non-infectious. As has been found for more conventional antiviral agents, the efficiency of inactivation is reduced by the presence of protective membranes and proteins [Druce et al., 1995; Marshall et al., 1995]. Our data indicate that cell-associated or skin-associated HIV-1 is more difficult to eliminate than free virus and HIV-1 inactivation is very temperature dependent. The persistence of infectious virus, albeit at reduced titre, for up to 5 days at 4°C suggests that the higher temperatures may accelerate the process of inactivation by improving the penetration of glycerol into tissue and replacement of water. It is clear that HIV-1 is more readily inactivated than non-enveloped viruses such as poliovirus which may take weeks to eliminate infectious virus [Marshall et al., 1995].

Using an experimental protocol similar to the current protocol used in skin banking, the initial incubation in 50% glycerol is unlikely to inactivate HIV-1. However we have shown that the subsequent steps of skin banking protocols in which skin is preserved in glycerol concentrations greater than 70% at room temperature or higher will effectively inactivate HIV-1 in experimentally infected skin. In current practice, the skin for banking is stored at 4°C for at least 2 weeks after the initial glycerolization and this subsequent 4°C storage would continue to reduce the titre of any residual virus. Certainly the elimination of infectious HIV-1 is faster than other viruses such as HSV. It is concluded from this data that skin from HIV-1 infected subjects is unlikely to be infectious after processing by the current glycerolization protocols as described here. In locations of high HIV prevalence glycerolization would provide higher margins of safety than could be obtained using alternative storage protocols such as cryopreservation.

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